RESEARCH LETTER

Effect of the association of reduced glutathione and ciprofloxacin on the antimicrobial activity in *Staphylococcus aureus*

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Abstract

We report the effect of glutathione and the role of reactive oxygen species (ROS), assayed by a nitro blue tetrazolium reaction, on the antibacterial action of ciprofloxacin, gentamicin and chloramphenicol in *Staphylococcus aureus* 22 resistant to ciprofloxacin and gentamicin, and in *S. aureus* ATCC 29213 sensitive to the above three antibiotics. The association of glutathione with ciprofloxacin or gentamicin significantly reduced the value of the minimum inhibitory concentration (MIC) in resistant *S. aureus* 22, measured using the macrodilution method, with a concomitant increase of intracellular ROS and a decrease of extracellular ROS. However, glutathione did not induce modifications in MIC or ROS generated by chloramphenicol. Furthermore, in the sensitive *S. aureus* ATCC 29213, the association of glutathione with ciprofloxacin, gentamicin or chloramphenicol did not induce any significant variations of MIC or ROS. There was a correlation between the stimulus of intracellular ROS and the decrease of MIC caused by exogenous glutathione. According to the results obtained, it is possible to modify the sensitivity of resistant strains of *S. aureus* by the addition of exogenous glutathione.

Introduction

Oxidative stress is involved in the action of antibiotics, such as aminoglycoside, which induce reactive oxygen species (ROS) by means of alteration of membrane proteins. Signaling through the envelope stress-response two-component system was demonstrated to be a key player. This signaling pathway was found for β-lactams and quinolones, which trigger hydroxyl radical formation by perturbation of the respiratory metabolism, with a subsequent increase of superoxide anion and release of ferrous iron (Kohanski *et al.*, 2008).

Generation of ROS can result in damage to the DNA, proteins and lipids. Related to this, we have previously shown that some antibiotics stimulate the production of ROS in different bacterial species (Albesa *et al.*, 2004), such as *Staphylococcus aureus* treated with ciprofloxacin (Becerra & Albesa, 2002; Becerra *et al.*, 2006).

Antioxidant systems prevent the uncontrolled formation of free radicals, and inhibit ROS and its reaction with biological structures. Increases in ROS, such as those that may occur during periods of oxidative stress, can be counteracted by regulatory molecules of the cell redox state, which trigger a homeostatic response to prevent cell injury. Antioxidant molecules, for example reduced glutathione, act against several oxidant compounds, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$) and reactive species of carbon. The small molecular reductants glutathione and cysteine can reduce a wide range of oxidized proteins, and protect against direct and indirect oxidation of lipid membranes and proteins as an adaptive response to increased basal oxidative damage caused by O_2^-. Glutathione can also be oxidized spontaneously in the presence of ROS and thus neutralize them by its antioxidant capacity. Furthermore, glutathione protects cells from the effects of the free radicals generated during metabolism and is considered to be a biological marker of the levels of antioxidant activity (Manfredini *et al.*, 2005; Cexiong *et al.*, 2009).

The aim of this work was to study whether the presence of exogenous glutathione can modify the susceptibility of *S. aureus* to different antibiotics, and to investigate any correlation with the oxidative stress.

Materials and methods

Determination of the minimum inhibitory concentration (MIC) in the presence of glutathione

The effect of exogenous glutathione on the inhibitory activity of ciprofloxacin, chloramphenicol and gentamicin was investigated in *S. aureus* ATCC 29213 and in clinical strain *S. aureus* 22, which were provided by Hospital Tránsito Cáceres de Allende (Buchardo 1250, Córdoba). The determination of the MIC for ciprofloxacin, gentamicin and chloramphenicol was performed using the broth macrodilution test, according to the Clinical and Laboratory Standards Institute (CLSI, 2006). To assess the activity of each antibiotic in the presence of glutathione, the bacterial suspension was incubated for 18 h at 35 °C with or without 10 mM glutathione, and with different concentrations of antibiotic. The lowest concentration of antimicrobial that prevented bacterial growth after 18 h of incubation was the MIC, both in the presence or in the absence of glutathione.

Determination of ROS by nitro blue tetrazolium (NBT) in *S. aureus* in the presence of antibiotics and glutathione

For the NBT reaction, 0.1 mL bacterial suspension ($OD_{600\,nm}$ 1.0) in phosphate saline buffer, pH 7, was incubated with 0.1 mL of the antibiotic and 0.5 mL of 1 mg mL^{-1} NBT for 30 min at 37 °C. Then, 0.1 mL of 0.1 M HCl was added and the tubes were centrifuged at 1500 ***g*** for 10 min, with the blue color of supernatants being measured at 575 nm (ROS extracellular). The separated pellets were treated with 0.6 mL dimethyl sulfoxide to extract the reduced NBT, and finally, 0.8 mL phosphate saline buffer was added and $OD_{575\,nm}$ was determined (ROS intracellular). These studies were carried out with suspensions of *S. aureus* ATCC, supplemented with 10 mM or in the absence of glutathione, and incubated with ciprofloxacin (0.033, 0.5 and 32 μg mL^{-1}) and gentamicin (0.125, 2 and 16 μg mL^{-1}). *Staphylococcus aureus* 22 was incubated with ciprofloxacin (0.5, 32 and 2048 μg mL^{-1}) and gentamicin (2, 16 and 2048 μg mL^{-1}). Determinations were also made in the absence of antibiotics (control).

Statistical analysis

The assays were performed at least in triplicate. Data were expressed as means ± SD and analyzed using Student's *t*-test. $P < 0.05$ was accepted as the level of statistical significance.

Results

In *S. aureus* ATCC 29213 sensitive to the three antibiotics assayed, the values of MIC obtained for ciprofloxacin, gentamicin and chloramphenicol were 0.5, 2 and 4 μg mL^{-1}, respectively. When the sensitivity to antibiotics was determined in the presence of glutathione, there were no significant changes in the MIC.

In *S. aureus* 22, the values of MIC were 32, 2048 and 8 μg mL^{-1} for ciprofloxacin, gentamicin and chloramphenicol, respectively, and according to the CLSI breakpoint categorization, this strain was resistant to ciprofloxacin and gentamicin. In the presence of glutathione, the MIC values of ciprofloxacin and gentamicin were significantly reduced. However, the addition of chloramphenicol and exogenous glutathione did not modify the susceptibility (Table 1).

In the NBT assay, an increase of intracellular ROS with respect to the basal without ciprofloxacin was observed in the sensitive *S. aureus* ATCC 29213. This effect was dose-dependent, with the increase of extracellular ROS with ciprofloxacin being lower than intracellular ROS (Fig. 1a). The resistant *S. aureus* 22 had less stimuli of intracellular ROS than the sensitive strain, but showed a higher extracellular ROS than *S. aureus* ATCC (Fig. 1b).

The oxidative stress associated with the increase in intracellular ROS was also observed with gentamicin in the sensitive strain *S. aureus* ATCC (Fig. 2a). No significant stimuli of intracellular ROS were found for resistant *S. aureus* 22, with 16 mg mL^{-1} of gentamicin being necessary to observe an increase in the extracellular ROS (Fig. 2b).

In the presence of glutathione and ciprofloxacin, we noted more stimuli of intracellular ROS than with ciprofloxacin alone, with resistant *S. aureus* 22 exhibiting a higher oxidative stress than in sensitive *S. aureus* ATCC 29213. On the other hand, extracellular ROS decreased with exogenous glutathione in both strains. Table 2 shows the percentages of alterations induced in ROS production with 10 mM of glutathione in the strains treated with 32 μg mL^{-1} ciprofloxacin and 16 μg mL^{-1} gentamicin. A 58.8–60% increase in ROS was caused by glutathione in the strain in which there

Table 1. Effect of exogenous glutathione in *Staphylococcus aureus* sensibility to antibiotics

	ATCC MIC (μg mL^{-1})	22 MIC (μg mL^{-1})
CIP	0.5	32
CIP+GSH	0.5	0.5*
GEN	2	2048
GEN+GSH	4	16*
CMP	4	8
CMP+GSH	4	4

*$P < 0.001$.
CIP, ciprofloxacin; CMP, chloramphenicol; GEN, gentamicin; GSH, glutathione.

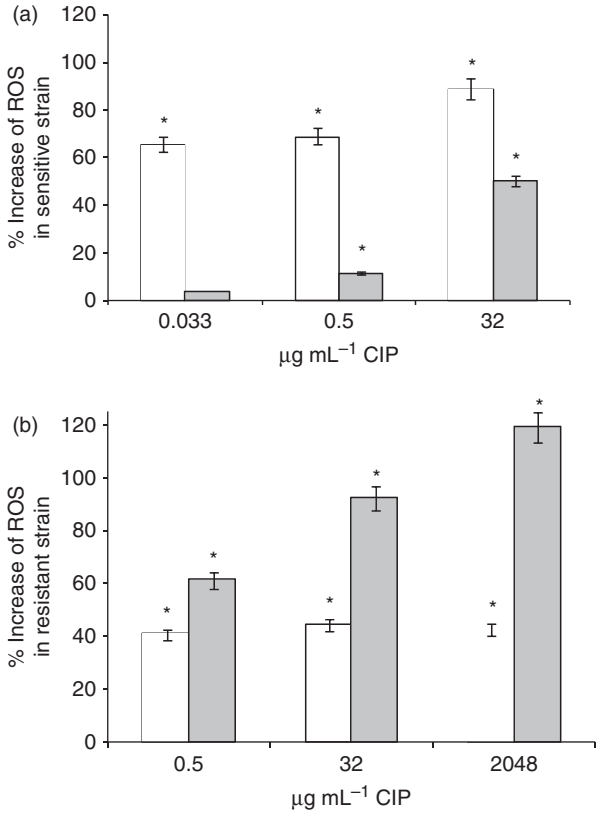

Fig. 1. Stimuli of ROS at different concentrations of ciprofloxacin. Percentages of intracellular (open bars) and extracellular (grey bars) increase of ROS generated in *Staphylococcus aureus* ATCC 29213 sensitive (a) and *S. aureus* 22 resistant (b) to ciprofloxacin. $^{*}P < 0.05$.

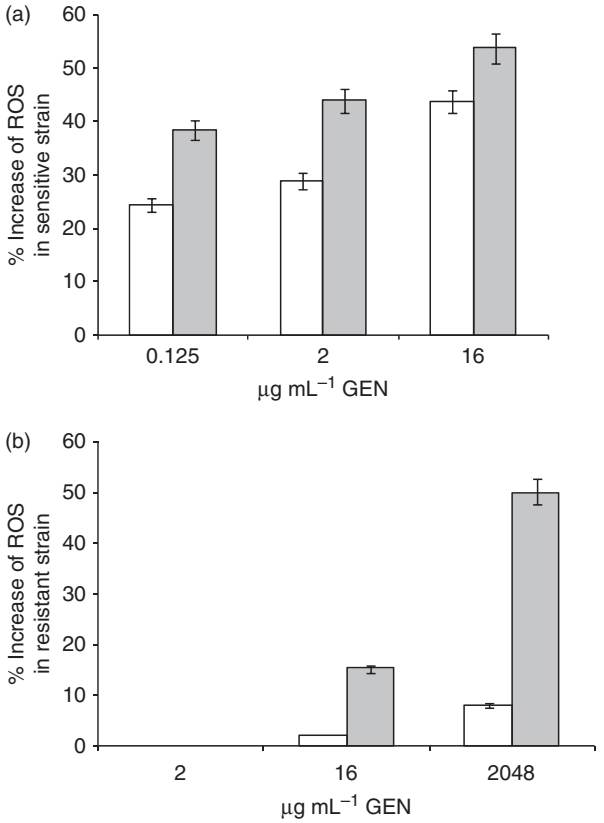

Fig. 2. Stimuli of ROS at different concentrations of gentamicin. Percentages of intracellular (open bars) and extracellular (grey bars) increase of ROS generated in *Staphylococcus aureus* ATCC 29213 sensitive (a) and *S. aureus* 22 resistant (b) to ciprofloxacin.

was a significant decrease in the MIC (resistant *S. aureus* 22), whereas in the sensitive strain, glutathione increases the production of ROS only by 12.8–16.6%, without any significant change occurring in MIC. There was a correlation between the stimulus of ROS and the decrease of MIC caused by exogenus glutathione. The glutathione stimulated intracellular ROS, even in strains without the antibiotic, and also increased the oxidative stress at all concentrations of the antibiotics assayed. However, this enhancement was more marked at the higher concentrations of both antibiotics (Figs 3 and 4). The exogenous glutathione decreased the extracellular ROS, up to a maximum of 86% in the two strains treated with ciprofloxacin, with similar results being obtained with gentamicin.

Discussion

It was previously shown that synthetic quinolone antibiotics promoted the formation of the hydroxyl radical that contributed to cell death (Kohanski *et al.*, 2007), and it was proposed that oxidative damage contributes to bactericidal cell death following gyrase poisoning with an oxygen-dependent death pathway appearing to amplify the primary effect on gyrase (Dwyer *et al.*, 2007).

Glutathione was chosen because it is a scavenger of ROS, which has been shown to be involved in protecting the cell either directly or indirectly. This might constitute an adaptive response to oxidative damage, which is known to increase in the presence of the antibiotic (Prinz *et al.*, 1997; Carmel-Harel & Storz, 2000; Pomposiello & Demple, 2002).

Compounds such as glutathione can rapidly cross the cell membrane, due to their hydrophobic nature, low molecular weight and the presence of specific transporters for these antioxidants in the cell membrane, thus allowing them to produce an antioxidant action in the cytosol (Parry & Clark, 2002; Zhang *et al.*, 2003).

A previous study conducted on *Escherichia coli* suggests that glutathione modulates the effect of antibiotics (Goswami & Jawali, 2007). These authors reported a reduction in MIC for ampicillin and penicillin, from 8 to 4 $\mu g\,mL^{-1}$ and from 64 to 48 $\mu g\,mL^{-1}$, respectively, which is not as marked as that found in our study for ciprofloxacin and gentamicin in *S. aureus*. According to our results, there exists the possibility of modifying the sensitivity of resistant strains of

Table 2. Increase of intracellular ROS and reduction of extracellular ROS by 10 mM of glutathione in *Staphylococcus aureus* treated with ciprofloxacin or gentamicin

	Intracellular ROS		
Strain	CIP (32 μg mL^{-1})	CIP+GSH	% Increase with GSH
ATCC	0.670 ± 0.150	0.756 ± 0.021	12.8
22	0.671 ± 0.007	1.074 ± 0.001*	60.0
	GEN (16 μg mL^{-1})	GEN+GSH	
ATCC	0.571 ± 0.041	0.666 ± 0.030	16.6
22	0.485 ± 0.010	0.768 ± 0.006*	58.8
	Extracellular ROS		
	CIP (32 μg mL^{-1})	CIP+GSH	% Reduction with GSH
ATCC	0.039 ± 0.002	0.022 ± 0.003*	33.6
22	0.050 ± 0.006	0.031 ± 0.003*	38.0
	GEN (16 μg mL^{-1})	GEN+GSH	
ATCC	0.066 ± 0.002	0.060 ± 0.003	9.1
22	0.030 ± 0.003	0.019 ± 0.001*	36.7

*$P < 0.05$ with respect to the samples without GSH.
CIP, ciprofloxacin; GEN, gentamicin; GSH, glutathione.

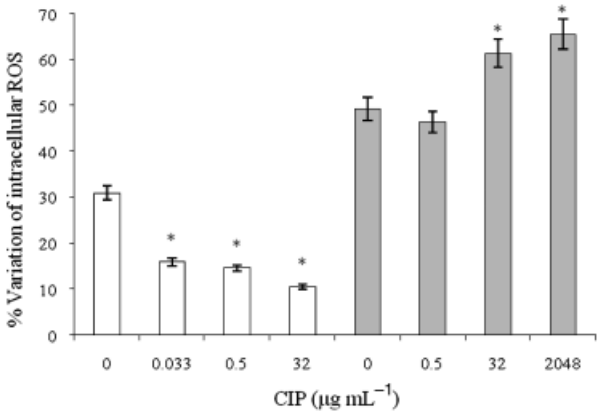

Fig. 3. Effect of glutathione on oxidative stress caused by ciprofloxacin. Increase of intracellular ROS induced by glutathione at different concentrations of ciprofloxacin in *Staphylococcus aureus* ATCC 29213 (open bars) and *S. aureus* 22 (grey bars). *$P < 0.05$ with respect to control without the antibiotic.

S. aureus by the addition of glutathione. These antecedents sustain the hypothesis of our work, which suggests that the antioxidants are useful to improve the bactericidal action of ciprofloxacin.

Considering that the antioxidant defense in *S. aureus* is transcriptionally regulated, and that the expression of *oxyR* genes occurs in response to external conditions via a

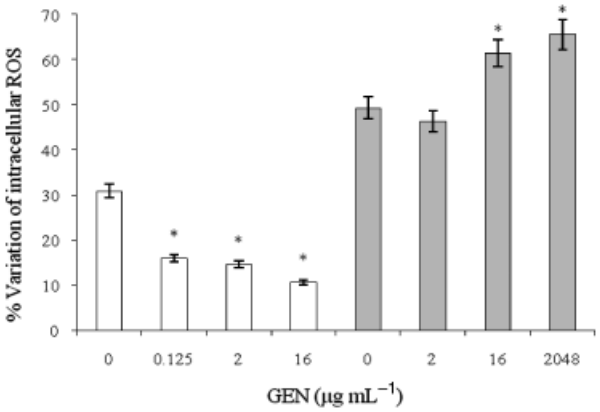

Fig. 4. Effect of glutathione on oxidative stress caused by gentamicin. Increase of intracellular ROS induced by glutathione at different concentrations of gentamicin in *Staphylococcus aureus* ATCC 29213 (open bars) and *S. aureus* 22 (grey bars). *$P < 0.05$ with respect to control without the antibiotic.

glutathione-dependent redox enzyme (Zheng *et al.*, 2001; Uziel *et al.*, 2004; Zeller & Klug, 2006; Roos *et al.*, 2007), it is reasonable to postulate that exogenous glutathione affects the defenses against the oxidative stress caused by antibiotics. In particular, our work shows that glutathione was able to modify the susceptibility of *S. aureus* to ciprofloxacin and gentamicin depending on the quantity of oxidative stress generated, which was higher in the resistant strain than in the sensitive one. These results could prove useful in future treatments combined with antibiotics.

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